

**RNA INTERFERENCE
(RNAi)
NUTS & BOLTS OF RNAi
TECHNOLOGY**

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Contents

Introduction	9
<i>Daniel J. Coughlin & David R. Engelke</i>	
Section I	
RNA Interference as an Experimental Tool in Invertebrates	17
Chapter 1	
Targeted Gene Silencing in Plants Using RNA Interference	19
<i>Sasha Preuss and Craig S. Pikaard</i>	
Chapter 2	
RNAi in <i>Caenorhabditis elegans</i>	33
<i>Maureen M. Barr and Juan Wang</i>	
Chapter 3	
Application and Analysis of RNAi in <i>Drosophila</i> Systems	53
<i>C. Lipardi, Q. Wei, and B. M. Paterson</i>	
Section II	
Short Interfering RNA as a Tool in Vertebrate Systems: Synthesizing the RNA	69
Chapter 4	
Gene Silencing by Synthetic siRNA Duplexes in Mammalian Cell Culture	71
<i>Agnieszka Patkaniowska and Thomas Tuschl</i>	
Chapter 5	
Strategies for Synthesizing Small Interfering RNA (siRNA)	89
<i>Q. Boese, W.S. Marshall, and S.A. Scaringe</i>	
Chapter 6	
Generating Long dsRNA, Individual siRNA and siRNA Cocktails <i>In Vitro</i>	113
<i>D. Brown, M.W. Byrom, R. Jarvis, V. Polatta, A.M. Cheng and L.P. Ford</i>	

Contents (continued)

Section III

Short Interfering RNA as a Tool in Vertebrate Systems:
Applications129

Chapter 7

Optimizing High Throughput RNAi-based Assays Using Transient
Transfection of Synthetic siRNAs in Cultured Mammalian Cells131
E. Krausz, A. Grabner, A. Kroenke, C. Sachse and C.J. Echeverri

Chapter 8

Subcellular Delivery and Detection of Small Interfering RNA169
Cynthia P. Paul

Chapter 9

Downregulation of Cellular Genes by PCR Products Expressing
siRNAs Mammalian Cells189
Daniela Castanotto, John J. Rossi, and Lisa Scherer

Chapter 10

Generating Adenoviruses for shRNA or siRNA Delivery205
Beverly L. Davidson

Chapter 11

Achieving Stable, Heritable Gene Silencing in the Mouse217
M. Carmell and G. Hannon

Index235

SECTION



**SHORT INTERFERING RNA AS A
TOOL IN VERTEBRATE SYSTEMS:
SYNTHESIZING THE RNA**

GENE SILENCING BY SYNTHETIC siRNA DUPLEXES IN MAMMALIAN CELL CULTURE

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4.1 Introduction

RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing mechanism, which is triggered by double-stranded RNA (dsRNA) and targets degradation of complementary mRNAs. Long dsRNA is cleaved by the endonuclease Dicer^[1,2] into 21-23 nucleotide (nt) duplexes with 2 nt 3' overhangs^[3] (Figure 4-1, Step 1). The resulting short interfering RNA (siRNA) duplexes are unwound and only one strand of each duplex is incorporated into the RNA-induced silencing complex (RISC)^[4,5] (Figure 4-1, Step 2). RISC is a multiple turnover RNA endonuclease complex^[6] (Figure 4-1, Step 5) and one of its components is a member of the Argonaute protein family^[5]. The single siRNA strand in the RISC guides the recognition of the complementary target mRNA and determines the position at which the mRNA is cleaved (Figure 4-1, Step 3). The target mRNA is cleaved within the region recognized by the antisense siRNA strand and the cleavage site is located opposite of the phosphodiester bond between 10th and 11th nt of the antisense strand counting from its 5' end^[5,7]. The mRNA fragments deprived of either cap structure or polyA tail, both required for mRNA stability, are subsequently degraded by cellular nucleases (Figure 4-1, Step 4). Consequently, the specific depletion of mRNA from the cell leads to a reduction of the corresponding protein, resulting in a knockdown phenotype.

The discovery of siRNAs as mediators of RNAi enabled their application in mammalian cell biology, bypassing unspecific cellular responses triggered by long dsRNAs^[8]. In most types of mammalian cells, dsRNA longer than 30 bp triggers dsRNA-activated protein kinase (PKR) and 2',5'-oligoadenylate synthetase (2',5'-AS), leading to blockage of protein synthesis initiation and to RNase L mediated RNA degradation, respectively (reviewed in:^[9]). These pathways are common to the interferon response which is ubiquitous in mammalian cells with the exception of oocytes^[10] and early embryonic cells^[2,11].

The first synthetic siRNA duplexes designed to mimic Dicer products were analyzed in *Drosophila melanogaster* embryo lysate^[3]. Soon thereafter they were shown to mediate silencing of endogenous genes in mammalian cell lines^[8]. Biochemical studies of the RNAi cleavage activity in *Drosophila* lysate and HeLa cytoplasmic extract indicated a high conservation of the RNAi mechanism between flies and mammals^[4,5].

This chapter discusses the design and application of synthetic siRNA duplexes for use in mammalian tissue culture. The use of chemically synthesized siRNA duplexes offers several advantages over alternative RNAi protocols presented in other chapters: (1) The sequence of synthetic siRNAs is well defined, in contrary to the siRNAs obtained by enzymatic processing.

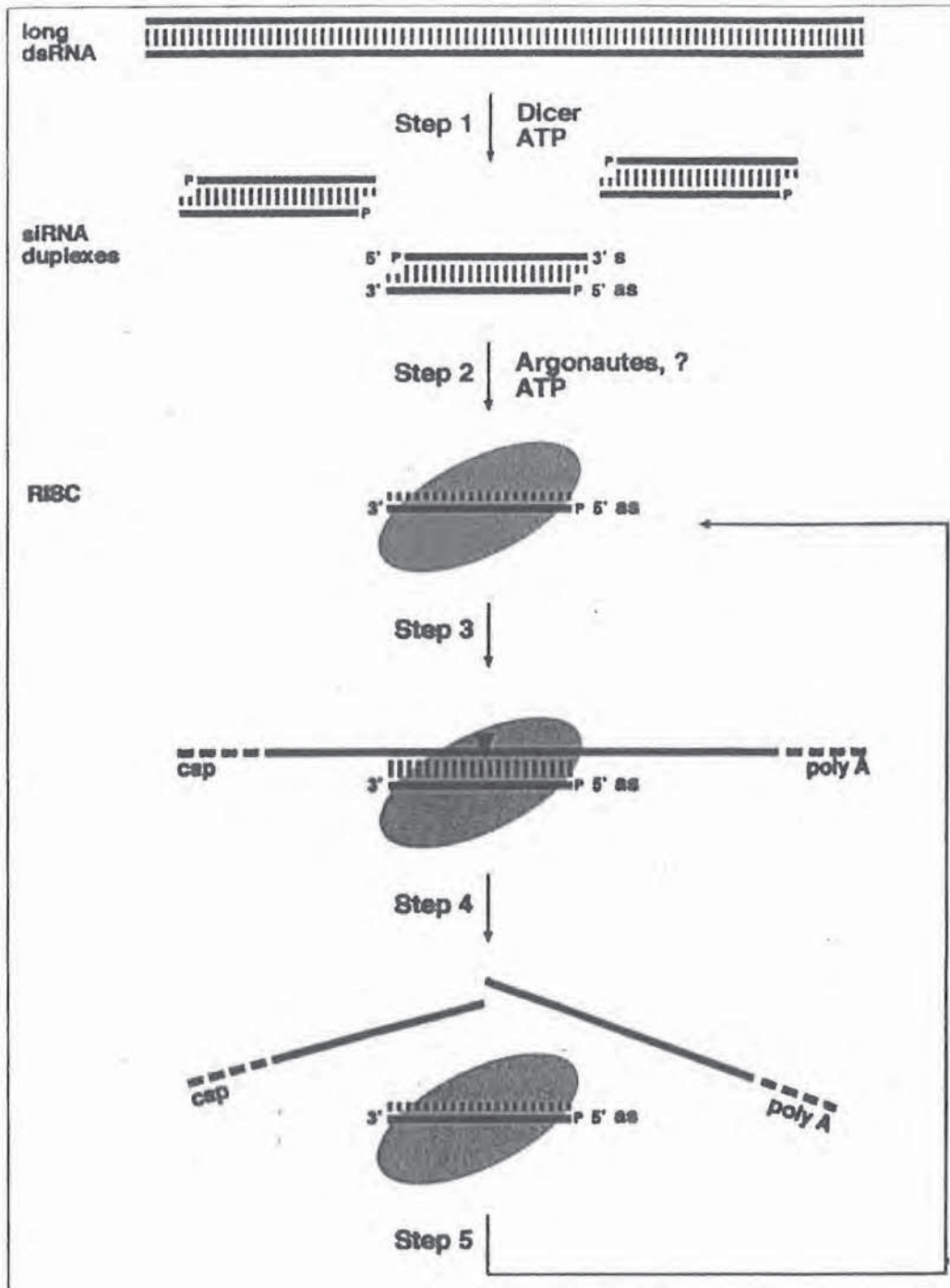


Figure 4-1: Outline of the RNAi mechanism. Long dsRNA is processed by the endonuclease Dicer into siRNA duplexes (Step 1). The duplexes are unwound and protein components of RISC assemble on one of siRNA strands (Step 2). RISC targets the complementary mRNA (Step 3). RISC cleaves the target (▼), releases the cleavage products (Step 4) and recycles (Step 5).

(2) Transfection of siRNA duplexes allows for the precise adjustment of the minimal siRNA concentration necessary to obtain a silencing effect. This is not easily accomplished with siRNA expression systems. (3) siRNA duplexes do not trigger the interferon response as observed for the siRNA expression systems^[12].

4.2 Structure

siRNA duplexes composed of two 21 nt RNA strands forming a 19 nt base-paired region with 2 nt 3' overhangs were found to be optimal for mediating RNAi in *Drosophila* lysate^[7] and in mammalian cell culture^[8]. In mammalian systems, however, RNAi can be induced with a wide variety of short dsRNA agents, such as 18-23 bp siRNA duplexes^[13,14] with or without 2 nt overhangs^[15] or 19-29 bp short hairpin RNAs (shRNAs)^[16]. siRNAs produced by Dicer cleavage contain 5'-phosphate and 3'-hydroxyl termini^[3,17]. Phosphorylation of the 5' ends, however, does not seem to be crucial for cell culture applications^[14,16,18] since free 5'-hydroxyl groups of RNA molecules are rapidly phosphorylated by endogenous kinases^[4,5].

RNAi can also be triggered by single-stranded siRNAs at high doses. Single-stranded siRNAs can associate directly with the RISC protein components bypassing the unwinding step of the siRNA duplex^[4,5]. Unmodified single-stranded RNAs, however, are generally too unstable for the routine use in cell culture^[16,18]. On the contrary, siRNA duplexes were shown to be unexpectedly resistant to nucleases contained in serum (stable for 48h in cell culture medium supplemented with serum^[19]) and in cytoplasmic extract (stable for at least 2h in HeLa cytoplasmic extract^[5]).

4.3 Nucleotide Composition

RISC activity is dependent on the sequence complementarity between the siRNA residing in the RISC and the cytoplasmic mRNA. The effect of mismatches depends on their nature and position with respect to the cleavage site. Single mismatches located in the middle of the guide siRNA are more inhibitory than mismatches at the ends of the siRNA strand^[16,20-22]. Additionally, mismatches close to the 5' end of the antisense siRNA are more disruptive than those at the 3' end^[18,23]. This is most likely related to the fact that the 5' end of the guide siRNA serves as a "ruler" by defining the position of mRNA cleavage^[3,5] (Fig.1, Step 3). It is probable, however, that some mismatches are better tolerated than others^[16] considering that RNA can support many different non-canonical base-pairings (e.g. G/U, U/G, C/A, A/C, U/U, C/U, U/C). The sequence of the antisense 3' overhang does not play a significant role in target recognition^[3]. In *Drosophila* lysate, only the 20th nt (counting from the 5' end of antisense strand) slightly contributes to the efficiency of RNAi^[7].

The overall G/C content of a siRNA sequence does not appear to influence silencing efficiency under standard siRNA application conditions, since duplexes with 30 to 80% G/C content effectively silenced various target genes^[14]. However, it is advisable to avoid more than 3 guanosine nucleotides in a row as they tend to form G-quartet structures^[14]. Recent studies in *Drosophila* have suggested that the relative strength of base-pairing at the 5' end of either siRNA strand partially determines which strand will be incorporated into RISC^[24,52]. A helicase activity involved in separating siRNA strands appears to preferentially unwind weak base-pairs (2 hydrogen bonds, A/U or U/A) rather than strong ones (3 hydrogen bonds, G/C or C/G). It is suggested that the siRNA strand for which the 5' end is most readily unwound, preferentially accumulates into RISC. The complementary strand is presumably degraded. This recent data suggests that siRNAs should be designed to have a lower G/C content at the 5' end of the antisense strand than at the 5' end of the sense strand. Adaptation of these recommendations may increase silencing efficiency as well as minimize off-target effects caused by the sense strand (see Selection).

4.4 Target mRNA

RNAi seems to occur predominantly in the cytoplasm^[4,5,25] and only a mature mRNA should be considered a target for siRNAs. Sequence information regarding mRNAs can be retrieved from public databases, e.g. NCBI (www.ncbi.nlm.nih.gov). When analyzing mRNA sequence data, it is important to consider the expression pattern of the gene of interest in order to assure that the target is present in the cells used for the experiment. One should also take into account the presence of any splice variants since siRNAs complementary to skipped exons will not be effective. Annotated single nucleotide polymorphisms (SNP) should be avoided since even a single nucleotide mismatch with the target sequence can drastically reduce the silencing efficiency of the siRNA (see Nucleotide Composition). The selected siRNA may still not be effective due to discrepancies between the annotated and the actual sequence of the target^[16].

The design of a powerful siRNA is still an empirical process, although with a reasonably high chance of success independently of the mRNA region being targeted (ORF or UTR)^[16]. siRNA duplexes show various silencing efficiencies, which can be explained by either the nucleotide composition of the siRNA duplex itself or by the targeted position within the mRNA. The positional effect can be attributed to the inaccessibility of the target site due to the secondary structure of mRNA or to binding of regulatory proteins. Prediction of the RNA secondary structure with software such as mfold^[26] does not correlate with the potency of an siRNA^[16,27,28], although some investigators like to suggest the opposite^[29]. Experimental data on mRNA accessibility from classical antisense

or ribozyme applications may be found useful when designing siRNAs^[21, 30-32]. However, this approach is neither time nor cost effective and does not guarantee success^[28, 31].

4.5 Selection

Each siRNA duplex should be tested *in silico* to avoid silencing of multiple genes (off-target effects). The antisense strand of the siRNA duplex can be checked by a nucleotide BLAST-search using appropriate parameters (www.ncbi.nlm.nih.gov/BLAST) to discard siRNA sequences with a high degree of complementarity to mRNAs other than the target. The number of mismatches to a non-target mRNA should be 3 or more^[33], located preferentially in the middle or towards the 5' end of the guide siRNA. Contiguous stretches of homology extending over 15 nt should be avoided, especially at the 5' end of the guide^[18,23].

To perform a search with the query sequence of 21 nt it is necessary to change the filters, word size and expect value threshold for the standard BLAST program. Low complexity filtering should be removed since it considers most of the short sequence insignificant, resulting in little or no query sequence remaining. The word size should be set to 7 letters, the minimal value for the algorithm used by this search. It is necessary to increase the expect value threshold (e.g. to 1000) since there is a high probability for a short sequence to occur by chance in the database. The expect value depends on the size of a database, so the threshold can be lowered when the search is restricted to a subset of the database (from the Description of BLAST services, NCBI). The parameters mentioned above are preset in the "BLAST search for short nearly exact matches" option.

The choice of the source database is very important, since it can considerably shorten the time of a performed search and further facilitate analysis of the results. The ideal database would be a non-redundant, comprehensive collection of mRNA sequences expressed in the cell line or tissue of the interest. Unfortunately, available resources are still far from that stage. For the moment, the optimal choices offered by NCBI are Reference Sequences (RefSeq) and UniGene databases. RefSeq is a non-redundant set of for the most part curated sequences, containing mRNA sequences and UniGene is a collection of gene-oriented clusters of mRNA and EST sequences. To facilitate the siRNA selection process there is some software available offering search interfaces for the most unique siRNA sequences within the mRNA of interest, e.g. a non-commercial website of the Whitehead Institute, MIT at: <http://jura.wi.mit.edu/bioc/siRNA/home.php>.

4.6 Verification

The possibility of facing some unspecific silencing effects cannot be excluded even when siRNAs are carefully selected. This has been recently demonstrated by microarray profiling studies^[23]. Not all off-target effects caused by siRNAs may be seen at the mRNA level. siRNAs can act like endogenously encoded miRNAs, which inhibit translation by hybridizing to partially complementary sequences in the 3'UTR of mRNAs^[34]. However, since miRNAs usually require several such target sites in the mRNA, translational inhibition is probably not a prominent off-target effect of siRNAs.

Any knockdown phenotype obtained via RNAi can be due to both intended and off-target effects. To avoid drawing wrong conclusions from the RNAi experiment, two or more different siRNA duplexes targeting the same mRNA should be independently tested for a reproducible phenotype^[33]. The ultimate proof for the specificity of a silencing experiment can be provided by a complementation approach (rescue experiment)^[33]. The silencing is considered to be specific, when the knockdown phenotype can be reversed by the introduction of a vector encoding the targeted gene. There are two approaches to a rescue experiment^[35]: (1) the siRNA duplexes can be directed against 3'UTR of the endogenous mRNA, which is lacking in the rescue vector; (2) silent mutations at the third position of a few codons can be introduced within the targeted region of the vector^[36]. In both cases, the wild-type phenotype should be observed when co-transfecting the rescue vector and siRNA specific for the endogenous copy of the gene. The knockdown phenotype should remain when co-transfecting the rescue vector and siRNA against a sequence common for the endogenous and introduced gene copy. For a well established co-transfection protocol see^[14]. An alternative to cDNA complementation is a rescue by introduction of the recombinant form of the depleted protein into the siRNA treated cells, e.g. by microinjection^[37].

4.7 Transfection

siRNA duplexes must be introduced into the cytoplasm in order to initiate silencing effects. For single cell study purposes, siRNA duplexes can be microinjected into the cells^[38,39] and the knockdown phenotype microscopically evaluated. For studying cell populations, however, introduction of siRNAs by either chemical transfection or electroporation are the methods of choice. Transfection efficiency and the level of cytotoxicity resulting from different siRNA delivery methods depend on the particular cell type being used^[40]. For this reason, the method and conditions of siRNA delivery should be optimized individually for all new cell types under investigation.

Chemical transfection is a convenient and widely used method for introducing siRNA duplexes into adherent cells. The most commonly used transfectants are cationic lipids, such as Oligofectamine (Invitrogen), Lipofectamine2000 (Invitrogen) and TransIT-TKO (Mirus) or calcium phosphate^[41]. There are however many other new reagents that are commercially available. For the standard transfection protocols see^[14] or follow the manufacturer's instructions. The ratio of siRNA duplex to transfection reagent is important for both the efficiency and toxicity of the transfection procedure. Excess of either of the components may have negative effects on the formation of the RNA/carrier complex or may result in the presence of either component in the non-complexed state. This may be toxic to the cells or decrease the uptake of siRNAs.

Electroporation is well suited for cells in suspension and can be readily adapted for adherent cells^[40-43]. This method delivers nucleic acids directly into the cytoplasm and does not require coupling of siRNAs with any carrier. The optimal parameters for the electroporation of siRNAs differ from those for plasmids by allowing the use of milder conditions that are less toxic^[41].

There is no universal transfection technique. Cells of different origin or morphology can vary with respect to cellular components involved in the processes that allow for the transition of siRNAs from the cell culture medium into the RISC. The duplex may fail to cross the cellular membrane, get trapped in endosomal structures or remain stably associated with the transfection reagent. Any of the above scenarios would contribute to reduction of transfection efficiency.

Titration of the siRNA concentration for both chemical transfection and electroporation is highly recommended, as it provides a graded readout of the silencing effect^[14]. This allows the identification of the minimal effective siRNA concentration, which reduces the probability of causing off-target effects^[20,44]. RNAi has been shown to occur at siRNA concentrations as low as 100 pM in the cell culture medium^[8]. Nevertheless, the minimal effective siRNA concentration as well as the kinetics of the silencing effect can vary for different transfection techniques and cell types^[41].

Every siRNA transfection experiment needs well-defined controls. The negative control aims to correct for the side effects that the transfection procedure exerts on the cells (see Silencing Effect). The siRNA duplex serving as a negative control should target a sequence that is not expressed in the transfected cell line, e.g. luciferase, GFP^[14]. Positive controls are used to assess the transfection efficiency and thus should provide a clear and quantifiable knockdown phenotype (e.g. Lamin A/C). When possible, it is recommended to quantify the

level of silencing for the positive control and the experimental sample by the same methods (see Silencing Effect). For a rough estimation of the transfection efficiency, it is possible to set up the positive control so that it produces an easy to spot phenotype that can be microscopically observed soon after transfection, e.g. β -actin depletion causing cellular blebbing^[45], Eg5 depletion resulting in loss of cell adhesion^[41,45] or any other essential protein knockdown driving cells into apoptosis.

Although commonly used cell transfection methods are very robust, they still require knowledge of some basic cell culture routine. To provide reproducible results, cells must be systematically split at the proper cell density and used only during early passages. Silencing experiments should be conducted at constant cell culture densities. Low cell densities during the transfection procedure can lead to an increase in cytotoxicity. Cell densities that are too high may lead to low transfection efficiencies. For chemical transfections, cells should typically be plated 24 hours prior to transfection at a density that results in confluency at the time of experimental readout. When choosing the initial cell density, it is important to consider that even low toxicity reagents impair the growth rate. Systematic monitoring of the cell morphology by phase contrast microscopy before and after transfection provides valuable information about the experiment.

4.8 Silencing Effect

The direct effect of RNAi is depletion of the targeted mRNA. The rate at which it occurs depends on the initial abundance and turnover rate of the particular mRNA. Reduction of the corresponding protein levels depends on the rate of mRNA depletion, initial protein concentration and its stability. The onset of the silencing effect depends on the rate of release of the siRNA duplex into the cytoplasm (see Transfection). Silencing effects monitored at the mRNA level are expected to appear earlier than those at the protein level. Using cationic lipid-based transfection, mRNA depletion is near completion within 24 hours, and reduction of protein level becomes readily detectable after 40 hours^[16]. For the electroporation based delivery, silencing effects may occur earlier, because of immediate delivery of siRNA into the cytoplasm^[43]. The silencing effect may last for 96 hours or longer^[14,40].

For validation of the silencing effect, standard cell, molecular biology and biochemistry methods are used. Methods for quantification of mRNA level include Northern hybridization, quantitative RT-PCR, real time PCR or branched DNA^[46]. For PCR based analysis, it is recommended to position the primers more than 50 nt upstream of the siRNA targeting site. The mRNA from knockdown and control cells should be purified using a polyA-specific mRNA isolation kits

in order to remove the (sometimes stable) target RNA 5' cleavage product prior to the amplification reaction^[16]. Immunodetection methods are used to quantify the level of protein, and can be performed on whole cells (immunocytochemistry, flow cytometry (FACS)) or cell lysates (Western analysis, immunoprecipitation (IP) and Enzyme Linked Immunosorbent Assay (ELISA)). Methods based on cell lysates rather than intact cells provide information about the average silencing effect for a cell population, while the whole cell readouts provide information about the nature of the silencing effect, i.e. what percent of the cell population shows the knockdown phenotype and to what extent.

4.9 Chemical Modifications

siRNA duplexes can be chemically modified and depending on the position of modification terminal and internal modification are distinguished. Terminal modifications are substitutions at the 5' or 3' position of a terminal ribose (*Table 4-1*). siRNAs are typically terminally modified to change their stability, uptake and cellular distribution or to serve as affinity tags in biochemical studies. Modifications designed to stabilize siRNAs against exonucleases are often nucleotide analogs that are connected to the siRNAs via non-natural 5',5' or 3',3' phosphodiester linkages (inverted nucleotides). Non-nucleotidic reagents are usually conjugated to the siRNAs via aminolinkers. When using siRNA duplexes linked to fluorophores it is important to keep in mind, that the cellular uptake of fluorescently labeled siRNAs is not necessarily correlated with their efficiency of gene silencing^[16].

While terminal modifications at both 5' and 3' end of the sense siRNA strand are very well tolerated^[5,15,16,47], it is more difficult to predict the effect for the antisense strand. Blocking of the 5' or the 3' end of the antisense siRNA has interfered with the function of siRNAs^[5,15,16,47]. In biochemical assays, it was shown that the 3' end of the antisense siRNA is permissive to modification^[5], but when some 3' modified antisense siRNAs were tested in cultured cells, they did not suppress the target gene^[16].

Internal modifications of siRNA duplexes comprise substitutions of the 2'-hydroxyl group of the ribose and modifications of the phosphate backbone (*Table 4-2*; for recent review see^[48]). Nucleotides with modified bases can also be introduced^[49]. Internal modifications are generally used to modulate the thermodynamic stability and conformation of the helix of siRNAs and their resistance against endonucleases. Among recently tested modifications are 2'-deoxy, 2'-O-methyl and 2'-fluoro-2'-deoxy substitutions at the ribose and phosphorothioate modifications of the backbone.

The most commonly used internal modification is substitution of two overhanging ribonucleotides at the 3' ends of siRNAs by deoxyribonucleotides, which enables reducing costs of RNA chemical synthesis with no effect on RNAi efficiency^[8,14,15,27]. 2'-Deoxythymidine overhangs (TT) have become the most widely used since their first successful application^[8]. The effect of modifications on RNAi efficiency depends on position and number of modified nucleotides within the base-paired region of the siRNA duplex^[15,19,49,50]. Modifications of the sense siRNA strand of the duplex interfere with the RNAi efficiency less than modifications of the antisense strand^[15,49]. Also modifications located in the 5' half of the antisense siRNA are more disruptive than those located in its 3' half^[49].

2'-fluoro-2'-deoxyribose is better tolerated than 2'-O-methylribose, presumably because the fluoride has properties similar to the replaced oxygen in the 2'-hydroxyl group. However, introduction of 2'-O-methyl modifications increase stability of siRNA duplexes in serum^[15] and prolong the silencing effect in the cell culture^[15,18]. A phosphorothioate backbone does not seem to impair the function of siRNAs, but the introduction of siRNAs with many sulfur-modified linkages may lead to cytotoxic effects^[16].

By careful selection of modifications and adjustment to the specific targeting sequence it should be possible to generate fully nuclease resistant yet active siRNAs, which may prove useful for *in vivo* applications. Conditions for optimal pharmacokinetic and pharmacodynamic distribution of siRNAs remain to be established. Conjugation of non-nucleotidic moieties to the siRNAs may be useful for modulating these parameters.

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Table 4-1.
Terminal modifications of siRNA duplexes.

Scale of the silencing effect as compared to the efficiency of unmodified siRNA duplex: +++++, > 80% of efficiency of unmodified duplex; +++, 60-80%; ++, 40-60%; +, 20-40%; -, modification rendering the duplex inactive.

Modification	Gene silencing	System	Reference
sense strand termini			
aminolinker	++++	HeLa extract	[5]
		HeLa	[15, 47]
puromycin or biotin	++++	HeLa	[47]
fluorescein	++++	HeLa	[16]
antisense strand 3' terminus			
aminolinker	++++	HeLa extract	[5]
		HeLa	[15]
puromycin or biotin	++++	HeLa	[47]
fluorescein	+++	HaCaT	[27]
fluorescein or Alexa488	-	HeLa	[16]
inverted deoxy abasic cap	++++	HeLa	[15]
antisense strand 5' terminus			
aminolinker	-	HeLa extract	[5]
		HeLa	[15, 47]
fluorescein	++++	HeLa	[16]
inverted deoxy abasic cap	-	HeLa	[15]

Table 4-2.
Internal modifications of siRNA duplexes.

Scale of the silencing effect as for the Table 4-1.

s, as - sense and antisense strand of siRNA duplex; 2'-Deoxy, 2'-OMe and 2'-OAl - deoxy, O-methyl and O-allyl modification of the 2'-hydroxyl group of ribose; LNA - locked nucleotides (2'-O,4'-methylene nucleotides); 2'FU, 2'FC - 2'-fluoro-2'-deoxyuridine and 2'-fluoro-2'-deoxycytidine; PS - phosphorothioate backbone;

* - effect depending on the position of modifications within siRNA duplex.

Modification	Position	Gene silencing	Cells	Reference
3' overhangs				
2'-Deoxy		++++	HeLa S3 HEK 293 COS-7 NIH3T3	[8]
			HaCaT	[27]
			HeLa	[14,15]
2'-OMe or 2'-OAl		++++	HaCaT	[18]
LNA		++++	HeLa	[19]
PT		++++	HaCaT	[18]
base-paired region				
2'-Deoxy	fully modified s	+	HeLa	[49]
	fully modified as	-		
2'-OMe	2-4 terminal nt of both strands	++++	HaCaT	[18]
	50% nt	+++ / ++ / +*	HaCaT	[50]
			HeLa	[15]
	fully modified s	+/-	HeLa	[15, 49]
	fully modified as	-		
2'-OAl	1 nt of both 5'ends	+++	HaCaT	[18]
LNA	1 nt of both 5'ends	++++	HeLa	[19]
	4-8 nt of both strands	++++ to - *		
2'FU and 2'FC	one or both strands	++++	293T CD4+ T	[51]
			HeLa	[16,19,49]
2'FU, 2'FC and 2'-Deoxy	3-13 2'-Deoxy nt in as strand	++++ / +++ / ++*	HeLa	[49]
2'FU, 2'FC and PS	3 PS linkages at both 3'ends	++++	HeLa	[16]
	fully PS modified as	+	HeLa	[49]
PS	25-50% linkages	++++	HeLa	[16, 19]
			HaCaT	[18]
			SW3T3	[16]
	both strands	+++ / ++	HeLa	[19, 49]

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